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GROWTH OF *STAPHYLOCOCCUS AUREUS* AND FUNGI IN BEEF SNACKS AT VARIOUS LEVELS OF WATER ACTIVITY

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Plain (type I) and spiced (type II) beef snacks are components of military rations. They were evaluated to determine the ability of S. aureus and selected fungi to grow in them at various water activity levels. S. aureus did not grow in type I beef snack at a_w 0.93 or lower, but it did grow in type II beef snack at a_w 0.92 and 0.94 with the elaboration of enterotoxin at a_w 0.94. Indigenous microflora grew in type I beef snack at a_w 0.93 , but not at 0.90 or lower. In type II beef snack, indigenous microflora grew at a_w 0.88 and above. Fungi did not grow in either type of beef snack at a_w levels between 0.71 and 0.88.

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PREFACE

For certain foods the combinations of temperature, wate activity (a_w, pH and redox potential (E_h), are capable of supporting the growth of Staphylococcus aureus and the elaboration of enterotoxin. This can occur at an a_w as low as 0.84. Beef Snacks Type I Plain and Type II Spiced are ready-to-eat, low moisture, cured foods not requiring refrigeration which are packaged in pouches under vacuum. The objectives of this study were twofold: (1) to define the a_w in Beef Snacks below which S. aureus cannot grow and produce enterotoxin, and (2) to determine the ability of selected fungi to grow in these products under reduced oxygen tension.

This is the second report completed this year on the effect of a_w on growth of microorganisms in Military Rations. The first study was of Tray Pack Cakes adjusted to various a_w levels and inoculated with Clostridium botulinum.

This study was performed under project number 1L162786AH99, by the Microbiology Branch, BioScience Division, Science and Advanced Technology Directorate, U. S. Army Natick Research, Development and Engineering Center, Natick, MA. The principal investigator was Edmund M. Powers.

The authors gratefully acknowledge the technical assistance of Mrs. Claire Lee for preparing the Staphylococcus aureus inoculum, and for carrying out the assays for S. aureus enterotoxin; and Robert Scott and Frank DiLeo of the Food Engineering Directorate for preparing the beef snacks.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

List of Figures

List of Tables

Growth of *Staphylococcus aureus* and fungi in beef snacks at various water activities

INTRODUCTION

Beef snacks are operational ration components and are ready-to-eat, low moisture, cured foods which do not require refrigeration. Proposed Military Specifications[1] require that the plain (type I) variety be produced at water activity (a_w) levels between 0.65 and 0.75, while the spiced, acidified (type II) variety is to be produced at slightly higher a_w levels between 0.68 and 0.81. Both products are dried by desorbtion to the desired levels of a_w by cooking in a smokehouse, rather than by the application of humectants. The pH is not prescribed[1] for beef snacks, but it is approximately 5.9 for type I and 5.7 for type II. The type II product is formulated with a spice/lactic acid mix which accounts for the slightly lower pH. Both types are formulated to initially contain 150 ppm nitrite. They are not processed to achieve sterility, but are shelf stable and inhibitory to the growth of indigenous microflora by virtue of the a_w, pH, nitrite, and low oxygen concentrations present. Although there is no public health concern at the above stated a_w values, an increase to a_w 0.86 could possibly support the growth of *Staphylococcus aureus*, which is the only foodborne pathogen known to grow at such a low a_w.[2]

The specific objectives of this study were to: (1) define the levels of a_w that prevent the growth of *S. aureus* in beef snacks produced for the Military, and (2) obtain a measure of the capability of fungi to grow in these products under anaerobic conditions at selected levels of a_w.

MATERIALS AND METHODS

Beef Snack Production

Beef snacks types I and II were formulated[1] and produced by the Food Engineering Directorate, U. S. Army R D & E Center, Natick, MA. The formulas are presented in the Appendix to this report. The products were processed and shaped in casings, frozen, and tempered to - 2.2°C, before slicing. The sliced beef snacks were then dried by cooking in a smokehouse until the target a_w levels were attained, as determined by measuring moisture content and relating it to a_w according to a standard curve. After removal of the beef snacks from the cookhouse, they were cooled to ambient temperatures and packaged under nitrogen in number 10 cans for delivery to the Microbiology Laboratory.

Experimental Samples

Four slices of beef snacks, totalling 29 grams, were placed into sterilized 211 x 101.5 metal cans. The center of each of the bottom three slices was inoculated with 7 µL (21 µL total) of either an *S. aureus* culture or a fungal culture, the preparation of which is presented below.

Samples of type I beef snacks having a_w levels of 0.75, 0.81, and 0.87 and type II beef snacks having a_w levels of 0.71, 0.83, and 0.88 were inoculated with fungi. Products at all a_w levels were inoculated with S. aureus. All samples were stored at 25°C under a vacuum of 26" of mercury in the metal cans. Cans were examined and cultured periodically for 90 days.

Preparation of Inocula

Staphylococcus aureus.

The S. aureus (A-100) inoculum was prepared as described by Lee et al.[3] The washed cells were diluted in Butterfield's phosphate buffer[5] so that 21 µL of the inoculum would deliver approximately 10^8 colony forming units (CFU) per gram of sample.

Yeast and Mold.

The mold inoculum was prepared by growing Aspergillus niger (QM 386) on Bacto Czapek Agar (Difco) at 25°C. Spores were scraped off the agar surface using sterile distilled water (SDW) + 0.1% Tween 80 and washed twice. The washed spores were resuspended in SDW and enumerated in a Petroff Hauser counting chamber.

The yeast inoculum was prepared by growing an unidentified yeast species, isolated from processed Meal, Ready-to-Eat (MRE) yellow apricots, on Bacto YM agar (Difco). The yeast was scraped off the agar surface in SDW and washed twice. The washed cells were resuspended in SDW and enumerated in a Petroff Hauser Counting Chamber.

The yeast cells and mold spores were mixed and diluted in SDW, so that 21 µL of the inoculum would deliver approximately 10^3 CFU of each per gram of sample.

Microbiological Analysis

Homogenates of the beef snack samples were prepared for analysis by stomaching[4] the entire contents (29 g) of each can in 200 mL of 0.1% peptone for two minutes. Serial tenfold dilutions of two samples were prepared in 0.1% peptone and 0.1 mL of each appropriate dilution was spread on the surface of each of two prepoured agar plates. S. aureus was enumerated on Baird-Parker Agar, incubated at 35°C for 48 hours. Only colonies typical of S. aureus were enumerated.[4] Yeast and molds were enumerated on Rose Bengal Streptomycin (35 mg/L) agar[5] incubated at 25°C for 5 days. Aerobic plate counts (APC) and anaerobic plate counts (ANPC) were determined on Tryptic Soy Yeast Extract (0.1%) agar plates incubated at 35°C for 48 to 72 hours.[4] Anaerobic incubation was accomplished in gaspak anaerobic jars (BBL) or in an anaerobic glove box (Coy Laboratory Products, Inc., Ann Arbor, MI).

Enterotoxin Assay.

Assays for staphyloccal enterotoxin A were performed on frozen and thawed samples of the original beef snack homogenates. Extraction from the homogenates and assay of the enterotoxin were performed by the methods of Fey and Pfister[4] using a SET-EIA Kit (Toxin Technology, Inc., 845 East Johnson St., Madison, Wisconsin 53703).

Measurement of Water Activity and pH

Water activity measurements of all samples were performed at 25°C before and after inoculation of the samples. Measurements were also taken periodically during storage at 25°C. The a_w of the beef snack was measured in a Beckman Hygroline a_w measuring station, model EEJA-3 (Beckman Industrial, Rosemount Analytical Division, 89 Commerce Road, Cedar Grove, NJ 07009). The measuring station is a humidity and temperature measuring device with three independent sensors to determine the equilibrium relative humidity and temperature of three samples at a time. The samples were equilibrated until constant readings were obtained. Saturated salt solutions were used to calibrate each sensor.

The pH of beef snacks was measured using an Orion Research Microprocessor Ionalyser/901 with an Orion 91-15 Combination electrode. The electrode was placed directly on the surface of the beef snack which had been moistened slightly with 0.5 mL of distilled water.

Chemical Analysis

Chemical constituents were measured using the procedures of the Association of Official Analytical Chemists:[7] moisture, item 24.002; protein, item 2.055; sodium chloride, item 18:034; fat, item 14.019 by the Physical Science Division, Science and Advanced Technology Directorate, Natick RD&E Center, Natick, MA.

RESULTS AND DISCUSSION

Enumeration of S. aureus and the APC of the inoculated and uninoculated samples of beef snacks are presented in Figures 1 and 2. Figure 1 shows that S. aureus inoculated into type I beef snacks did not grow at a_w 0.93 or lower. The minimum a_w for growth of S. aureus was not determined since a_w levels higher than 0.93 were not studied. At a_w 0.87 and 0.90, S. aureus counts decreased over time while at a_w 0.93 they remained fairly constant for 90 days. In the inoculated samples, the APC represented the recovery of S. aureus since the two counts were approximately the same. Appreciable growth of the indigenous microflora in Type I beef snack occurred at a_w 0.93, but not at a_w levels of 0.87 and 0.90, as indicated by the increase in the APC of the uninoculated control samples at 28 and 90 days.

The growth of S. aureus in Type II beef snack is shown in Fig. 2. The populations of S. aureus showed an increase at a_w 0.92 and 0.94, after 28 and 90 days and after 14 and 60 days, respectively. There was no growth of S. aureus at a_w levels of 0.88 or 0.89. The minimal a_w for

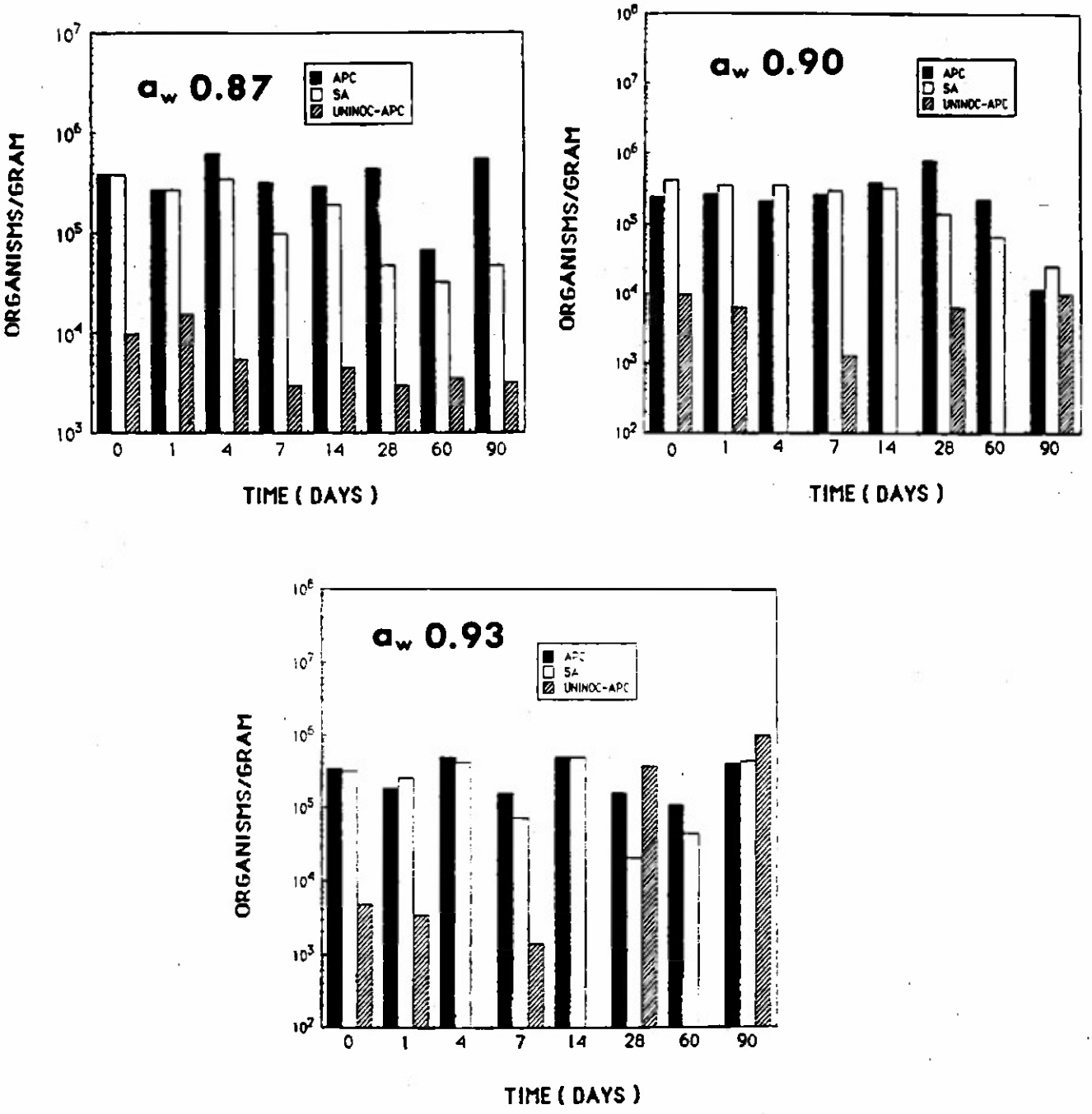

Figure 1. The aerobic plate count (APC) and the *Staphylococcus aureus* count (SA) in the inoculated samples, and the uninoculated control APC (UNINOC-APC) for type I beef snacks.

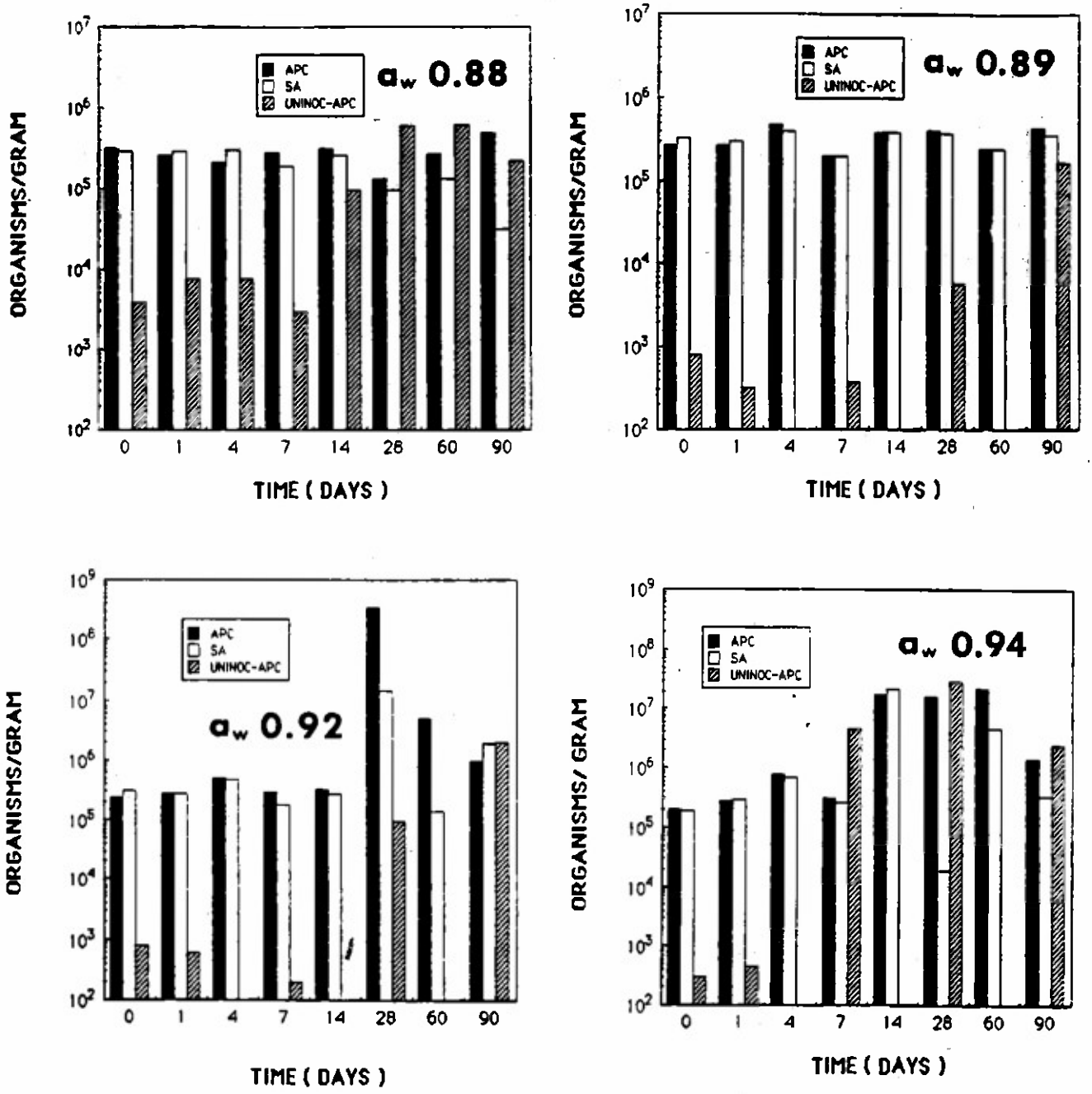

Figure 2. The aerobic plate count (APC) and the *Staphylococcus aureus* count (SA) in the inoculated samples, and the uninoculated control APC (UNINOC-APC) for type II beef snacks.

growth of S. aureus in Type II beef snacks is therefore between a_w 0.89 and 0.92, but products having an a_w between these two values were not available. The failure of S. aureus to grow under vacuum at a_w below 0.90 was not unexpected since a previous study reported that anaerobic growth of S. aureus in precooked bacon was not observed below a_w 0.90 when incubated at 37°C.[3] Under aerobic conditions however, S. aureus grew in the precooked bacon at a a_w as low as 0.84. Therefore, temperature, as well as the presence or absence of oxygen, influences the minimal a_w for growth of S. aureus. The indigenous microflora in Type II beef snacks grew at all four a_w values presented in Fig. 2 as indicated by the increase in the APC of the uninoculated control samples. Growth was evident at 14 days at a_w 0.88, at 28 days at a_w 0.89 and 0.92, and as early as 7 days at a_w 0.94. Only the highest a_w values are presented in Fig. 1 and Fig. 2 because there was no bacterial growth at lower a_w values studied which included a_w values as low as 0.75 for type I and 0.71 for type II. This is further illustrated in Fig. 3, which shows the decline of S. aureus at these lower a_w levels. Anaerobic plate counts were performed on all samples studied, but are not reported since they were approximately the same as the APC values.

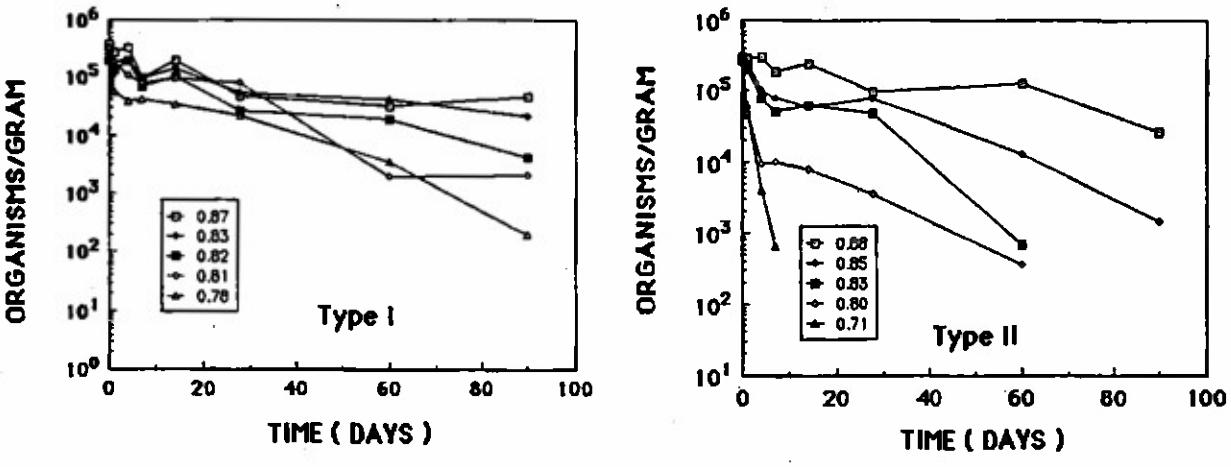

Figure 3. Decline in *S. aureus* at different water activities in type I and type II beef snacks.

Staphylococcal enterotoxin A (SEA) was detected only in type II beef snacks at a_w 0.94, after 14 days of incubation as shown in Table 1. No enterotoxin was detected in later samples taken at this a_w. Whereas previous studies[2, 3] have attempted to relate ability to produce enterotoxin to cell concentration, the detection methods used were sensitive to 0.1 µg/mL. The assay method used in the present study detects 0.1 to 1 ng/mL of staphylococcal enterotoxin. Therefore, it is unlikely that enterotoxin was produced in other samples tested (Table 1). Negative assays may have also been due partly to deterioration of SEA caused by freezing and thawing of the sample. This was demonstrated when the assay of the sample which contained 1.77 ng/g was repeated after the

TABLE 1. Enterotoxin A production by Staphylococcus aureus in beef snacks at selected water activity levels during storage at 25°C.

		Enterotoxin A (ng/g beef snack)			
		Days			Maximum CFU/g
Type	a_w	7	14	90	
I	0.85	--[a]	--	0	3 X 10^5
	0.87	--	--	0	3 X 10^5
	0.90	0	0	0[b]	3 X 10^5
	0.93	0	0	0[b]	3 X 10^5
II	0.89	0	0	0[b]	3 X 10^5
	0.92	0	0	0[b]	1 X 10^7
	0.94	0	1.77	0[b]	2 X 10^7

[a] Not tested

[b] Enterotoxin A was also undetected in 90 day samples taken directly from cans in stoage at 25°C.

sample was frozen and thawed. The enterotoxin concentration in this case was reduced to 0.164 ng/g of beef snack.

The isotherm in the hysteresis curve of Fig. 4 shows that the relationship between a_w and percent moisture was virtually the same for both types of beef snacks. Similarly, the relationship between water activity and brine concentration listed in Table 2 and illustrated in Fig. 5 is seen to be the same for type I as it is for type II.

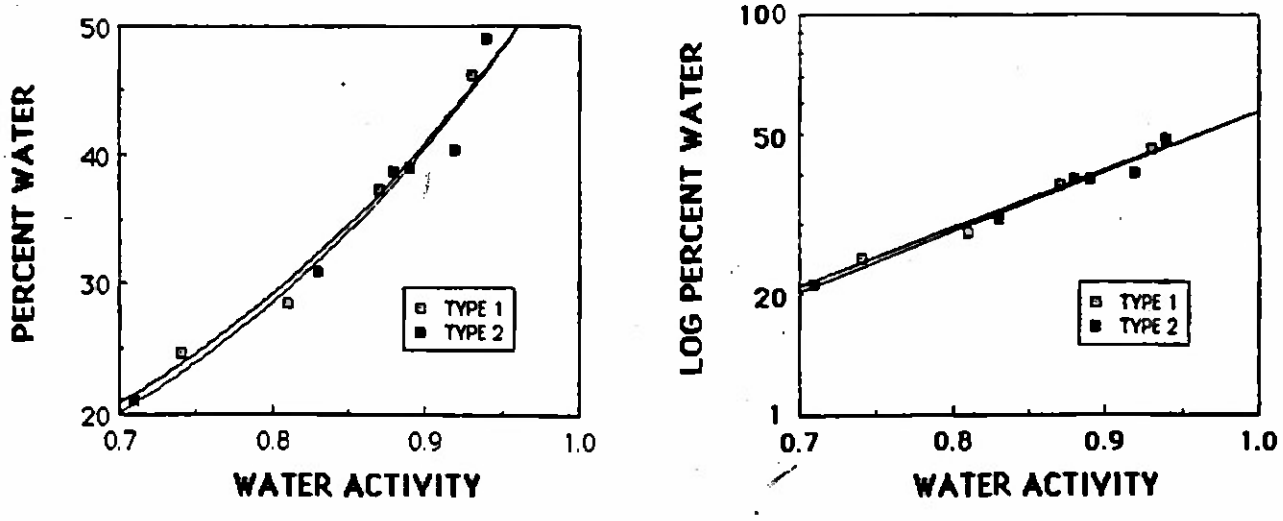

Figure 4. Isotherms of types I and II beef snacks.

TABLE 2. Proximate composition and pH of beef snacks types I and II adjusted to selected water activities (a_w).

Beef snack Type	a_w	Percentage by weight						
		Moisture	Salt	Protein	Fat	M/S	Brine	pH
I	0.74	24.66	5.53	51.47	12.45	4.45	22.4	5.87
	0.81	28.53	5.21	48.54	12.61	5.47	18.3	5.80
	0.87	37.42	4.40	37.42	12.56	8.50	11.7	5.87
	0.89	37.13	3.76	41.71	11.11	9.87	10.1	5.61
	0.90	40.86	3.81	40.32	10.11	10.72	9.3	5.57
	0.93	46.12	3.35	36.41	9.81	13.76	7.3	5.58
II	0.71	21.05	5.48	51.40	12.78	3.84	26.0	5.67
	0.83	30.96	4.82	44.44	12.18	6.42	15.6	5.70
	0.88	38.73	4.28	39.66	10.94	9.04	11.0	5.92
	0.89	39.06	4.14	43.44	10.00	9.43	10.6	5.73
	0.92	40.44	4.03	43.76	9.89	10.03	10.0	5.75
	0.94	49.14	3.24	36.67	8.68	15.6	6.6	5.74

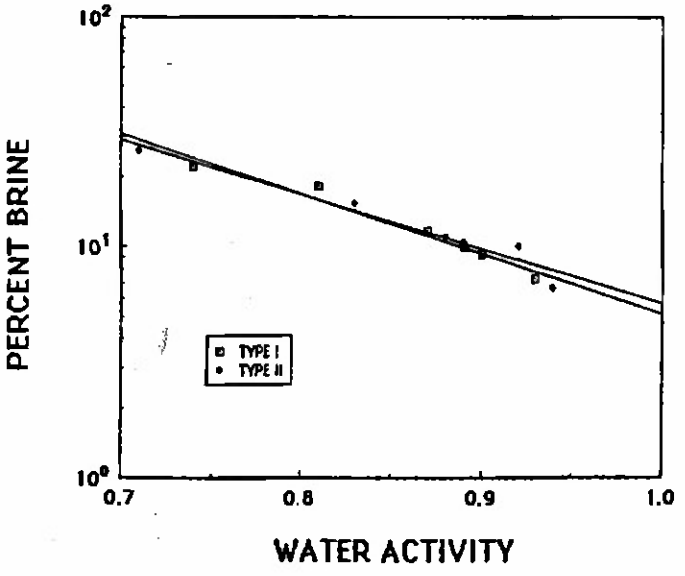

Figure 5. Relationship between water activity and brine concentration in type I and type II beef snacks.

Therefore, differences in bacterial growth between the two types of beef snacks probably cannot be ascribed to small differences in moisture or salt content at equivalent levels of a_w. However, identical levels were not available for both types of beef snacks. Other investigators have reported on the effects of small differences in a_w, pH, and salt concentrations on inhibition of growth. Denny et al.[6] reported that C. botulinum produced toxin at a_w 0.955, but not at 0.950 in canned bread. Tanaka et al.[7] reported that toxin was formed in one cheese spread but not in another which differed in pH only by 0.03 units, in moisture by 4.71%, and in NaCl plus phosphate by 0.147%. Small changes in formulations are especially critical when moisture levels are high or when conditions are on the borderline for growth or toxin production.

Any expected inhibition of microbial growth by spices was evidently not a factor. Both types of beef snacks contained red and black pepper, but type II, the less microbiologically inhibitory product, contained a greater variety of spices (see formulation in Appendix). The nitrite concentration was the same in both types of product.

To determine if selected strains of yeast or mold could grow or survive on beef snacks, a_w levels were selected for inoculation that would represent the high, middle, and low range of the levels of a_w initially studied. Beef snacks selected later for study at a_w levels greater than 0.88 were not inoculated with the yeast and mold. Table 3 shows that the yeast and mold not only failed to grow, they did not survive long at a_w levels ranging from 0.71 to 0.88 in the two products (types I and II). The mold appeared to be more hardy than the yeast under the vacuum established in the cans and in type II, survived for a maximum of 90 days at a_w 0.83. At all other a_w levels studied, survival was for only 28 days or less. The longest time that yeast survived was 28 days and the shortest time was 4 days (a_w 0.75 in type I).

Table 3. Recovery and survival of yeast and mold experimentally inoculated into beef snacks at selected water activities

Beef Snack Type[b]	a_w	Yeast			Mold		
		CFU[a]/g			CFU/g		
		Initial	Final	Days Survived	Initial	Final	Days Survived
I	0.87	7×10^3	<40	14	6×10^3	<40	14
	0.81	5×10^3	<40	7	4×10^3	<40	14
	0.75	8×10^3	<40	4	6×10^3	<40	14
II	0.88	4×10^2	<40	28	8×10^2	<40	14
	0.83	4×10^2	<40	7	4×10^3	<40	90
	0.71	5×10^2	<40	7	6×10^2	<40	28

[a] Colony forming units
[b] Stored at 25°C for 180 days

The data indicated that the yeast and mold selected for inoculation into the beef snacks would not grow or survive when sealed under vacuum at a_w levels permitted by the proposed Military Specification for beef snacks.[1] Permissible a_w levels range from 0.65 to 0.75 for type I, and from 0.68 to 0.81 for type II beef snacks. However, in the event of a package failure which exposes the products to oxygen as well as ambient moisture levels, mold growth will occur as it did when uninoculated beef snacks at the above a_w levels were deliberately exposed by the authors to ambient air. Therefore, to prevent fungal spoilage it is essential to maintain the integrity of the package by restricting both oxygen and moisture.

A better understanding of the interactive effects of these factors in a food such as beef snacks is still needed so that formulation and processing parameters could then be logically modified for increased acceptability while still ensuring microbiological safety. While raising the a_w limit of beef snacks may improve their acceptability, further improvements and greater acceptance might be possible by modifying other factors and components of the beef snack formulation, such as pH, salt, and nitrite concentrations. For example, pH limits are not specified for beef snacks. By lowering the pH a higher moisture product may be produced, which would possess equivalent safety. Additionally, ascorbate might be included, or nitrite might be reduced or omitted if the a_w and pH are low enough, thus reducing concern about the use of nitrite. By incorporating additional water binding agents it would be possible to reduce the salt content and still retain the desired a_w and safety.

CONCLUSIONS AND RECOMMENDATIONS

S. aureus did not grow in Type I beef snack at a_w 0.93 or lower, but it did grow in Type II beef snack at a_w's 0.92 and 0.94. At a_w 0.94 the growth was accompanied by the production of enterotoxin.

The indigenous microflora grew in Type I beef snack at a_w 0.93, but not at 0.90 or lower. In the Type II beef snack, growth of the indigenous microflora occurred at a_w's 0.88, 0.89, 0.92, and 0.94.

Growth of representative fungi did not occur in Type I or II beef snacks at a_w levels between 0.71 and 0.88.

These results indicate that a_w limits specified for type I and type II beef snacks (a_w 0.65 - 0.81) will provide microbiological stability.

Further research to investigate the interactive relationships between a_w and other factors and components of the formulation is recommended. Since the growth of the indigenous microflora occurred in both types of beef snacks and especially in type II at a_w 0.88, it would be of interest to determine the limiting a_w of type II beef snack that would prevent the growth of the indigenous microflora. Identification of these microorganisms possessing an ability to survive and grow in the beef snacks after the cooking process is of great interest. This could be accomplished by examining the indigenous microflora of beef snacks and challenging their ability to grow at a_w levels ranging from 0.80 to 0.88.

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APPENDIX

PREPARATION OF BEEF SNACKS

a. Type I formulation.

Ingredients	Percent by weight
Beef, flaked or ground	97.195
Salt	2.09
Pepper, black, ground	0.10
Pepper, red, ground	0.05
Sodium ascorbate	0.05
Sodium nitrite ($NaNO_2$)	0.015 (150 ppm)
Liquid smoke	0.50

b. Type II formulation.

Ingredients	Percent by weight
Beef, flaked or ground	95.6
Salt/nitrite mix	2.1
Spice/lactic acid mix	1.8
Liquid smoke	0.5
Salt/Nitrite Mix	
Salt	99.3
Sodium nitrite ($NaNO_2$)	0.7
Spice/Lactic Acid Mix	
Paprika	40.0
Sugar	30.6
Lactic acid, encapsulated	8.8
Pepper, black, ground	5.5
Ascorbic acid, encapsulated	3.9
Fennel, ground	3.3
Pepper, red, ground	2.8
Garlic, powdered	2.8
Coriander, ground	1.7
Allspice, ground	0.6